

Mutations in Calcium-binding Epidermal Growth Factor Modules Render Fibrillin-1 Susceptible to Proteolysis

A POTENTIAL DISEASE-CAUSING MECHANISM IN MARFAN SYNDROME*

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Most extracellular proteins consist of various modules with distinct functions. Mutations in one common type, the calcium-binding epidermal growth factor-like module (cbEGF), can lead to a variety of genetic disorders. Here, we describe as a model system structural and functional consequences of two typical mutations in cbEGF modules of fibrillin-1 (N548I, E1073K), resulting in the Marfan syndrome. Large (80–120 kDa) wild-type and mutated polypeptides were recombinantly expressed in mammalian cells. Both mutations did not alter synthesis and secretion of the polypeptides into the culture medium. Electron microscopy after rotary shadowing and comparison of circular dichroism spectra exhibited minor structural differences between the wild-type and mutated forms. The mutated polypeptides were significantly more susceptible to proteolytic degradation by a variety of proteases as compared with their wild-type counterparts. Most of the sensitive cleavage sites were mapped close to the mutations, indicating local structural changes within the mutated cbEGF modules. Other cleavage sites, however, were observed at distances beyond the domain containing the mutation, suggesting longer range structural effects within tandemly repeated cbEGF modules. We suggest that proteolytic degradation of mutated fibrillin-1 may play an important role in the pathogenesis of Marfan syndrome and related disorders.

The epidermal growth factor (EGF)¹-like module is a widely used structural element in many extracellular matrix proteins, blood proteins, and membrane-bound proteins (1). A subset of this module contains an amino acid pattern ((D/N)X(D/N)(E/Q)X_m(D*/N*)X_n(Y/F); *m* and *n* are variables, an asterisk (*) denotes potential β -hydroxylation (2)), that mediates calcium binding (cb) in the N-terminal pocket of the module. These cbEGF modules are often arranged in repeating tandem arrays (1). While isolated cbEGF modules without adjacent units often show relatively low affinities for calcium in the 1–5 mM range

(2–4), tandemly repeated cbEGF modules display much higher affinities (5, 6), probably due to stabilization effects at the N-terminal end of each cbEGF module (7) or to longer range stabilizing effects (8). Calcium binding to cbEGF modules has been suggested to play a functional role in protein-protein interactions (9, 10) and to protect these modules against proteolytic cleavage in fibrillin-1 (11) or fibulin-1 and -2 (12). The importance of cbEGF modules is emphasized by genetic mutations changing amino acid residues in these modules that result in Marfan syndrome (fibrillin-1 (13)), congenital contractural arachnodactyly (fibrillin-2 (14)), Protein S deficiency (Protein S (15)), hemophilia B (Factor IX (16)), familial hypercholesterolemia (LDL receptor (17)), and “CADASIL” causing cerebral arteriopathy and leukoencephalopathy (Notch3 (18)).

In this report we focus on the structural and functional effects of two different mutations within cbEGF modules of fibrillin-1 causing varying forms of the Marfan syndrome, a dominantly inherited disorder characterized by cardiovascular, skeletal, and ocular abnormalities. Fibrillins are major integral components of supramolecular fibrillar structures called microfibrils. Two highly homologous members, fibrillin-1 and fibrillin-2, each contain 43 cbEGF modules that are dispersed among other structural motifs in groups of 1–12 units over the entire molecule (19, 20). From the 137 mutations in fibrillin-1 published in the Marfan data base (13), 102 occur within cbEGF modules. 26 of these mutations are predicted to cause major disruptions of the fibrillin-1 molecule by introducing frameshifts or by creating stop codons, and 64 different mutations (47% of total number, 12 redundant mutations on the protein level) cause a change of a single amino acid residue in cbEGF modules. These data underscore the importance of cbEGF modules for the biological integrity of fibrillin-1.

The point mutations in cbEGF modules can be classified into three groups: (i) mutations eliminating or generating cysteine residues, (ii) mutations of residues directly involved in calcium binding, and (iii) mutations of other residues. The effects of these mutations are believed to alter either the natural folding and/or calcium binding of these modules. For a few fibrillin-1 mutations, the structural/functional effects of small recombinant or synthetic peptides have been reported. For instance, G1127S and R1137P led to misfolding of cbEGF13 (21, 22), whereas N2144S did not cause an observable structural change of cbEGF32 or the adjacent cbEGF33, but resulted in a reduced affinity for calcium in cbEGF32 (23). How a protein phenotype, “misfolded cbEGF” or “reduced calcium affinity,” can consequently result in the clinical phenotype of Marfan syndrome is not known.

In this study, we demonstrate that two Marfan mutations N548I and E1073K in cbEGF modules render recombinantly expressed polypeptides susceptible to proteolysis. Sensitive

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¹ The abbreviations used are: EGF, epidermal growth factor; cb, calcium binding; TBS, Tris-buffered saline; wt, wild-type; bp, base pair(s).

sites are mapped in detail close to the site of the mutations. Structural analyses demonstrated small changes introduced by the mutations. These results indicate that subtle conformational changes in fibrillin-1 are sufficient to precipitate the cascade of biological events leading to disease.

EXPERIMENTAL PROCEDURES

Production of Recombinant Cell Clones—The construction of an episomal plasmid to express wild-type rF18 (Asp⁹¹⁰-Val¹⁵²⁷; rF18-wt) was described in detail previously (pCEPSP-rF18H (6)). To introduce the neonatal Marfan mutation G3217A (24, 25) leading to amino acid substitution E1073K, plasmid pCis-rF18H (6) and two complementary oligonucleotides harboring the mutation (underlined), DR128 (5'-GGAAC-TGCACAGACATTGACAAATGCCGCATATCTCCTGACC-3') and DR129 (5'-GGTCAGGAGATATGCCGCATTGTCAATGTCTGTGCA-GTTCC-3'), were used in the QuikChange mutagenesis procedure as instructed by the manufacturer (Stratagene). An 1884-bp *NheI*-*NotI* fragment was cut out from the resulting plasmid and subcloned into a *NheI*-*NotI* restricted pCEP4/γ2III4 (26), to yield plasmid pCEPSP-rF18-E1073K. The mutation and the entire insert was verified by DNA sequencing.

A new plasmid coding for Asp⁴⁵¹-Lys¹⁰²⁷ of fibrillin-1 (rF45-wt) was generated by subcloning a 3261-bp *NheI*-*NotI* fragment from pCEPSP-rF20 (6) into pBluescript II SK(+) (Stratagene), resulting in pBS-rF20a. A 222-bp *Bsu36I*-*SacI* fragment generated from a polymerase chain reaction amplification product with template HFBN8 (19) and primers 8FS (5'-TCAAGAAATTAAGGAACACA-3') and DR126 (5'-TCGTAGAGCTCGCGGCCGCTTACTATTGGAAGAAAGGCTTTCCA-3') were ligated with the *Bsu36I*-*SacI* fragment generated from pBS-rF20a, resulting in plasmid pBS-rF45. Finally, the 1745-bp *NheI*-*NotI* fragment from pBS-rF45 was subcloned into pCEP4/γ2III4 vector (pCEPSP-rF45). To introduce the mutation A1643T (27) into pCEPSP-rF45, site-directed mutagenesis was performed using pBS-rF45 and the complementary primer pair DR135 (5'-GGACGCTGCATCATCACAGATGGCAGTTTTC-3') and DR136 (5'-GAAACTGCCATCTGTGATGATGCAGCGTCC-3') by the QuikChange procedure as suggested by the supplier (Stratagene). The region between restriction sites *NheI* and *NsiI* (721 bp) including the mutation was verified by DNA sequencing and then subcloned into wild-type pBS-rF45. The entire 1745-bp *NheI*-*NotI* insert was then subcloned into pCEP4/γ2III4 resulting in pCEPSP-rF45-N548I. Transfection of 293-EBNA cells with the expression plasmids and selection with hygromycin B was performed as described in detail previously (6).

Production, Purification, and Characterization of Recombinant Polypeptides—Production of conditioned medium, and purification of rF18-wt and rF18-E1073K was performed as described previously for rF18 (6). For purification of rF45 and rF45-N548I, about 2–3 liters of serum-free medium were concentrated by ultrafiltration (30 kDa cutoff) to ~80–100 ml, dialyzed against 20 mM Tris-HCl, pH 8.6, and passed over HiTrapQ (5 ml; Amersham Pharmacia Biotech) equilibrated in the same buffer. Bound proteins were eluted with a gradient of 1 mM NaCl/ml. Fractions containing rF45 or rF45-N548I were pooled and concentrated by ultrafiltration (10 kDa cutoff) to ~1.5 ml and passed over a Superose 12 gel filtration column (100 ml; Amersham Pharmacia Biotech) equilibrated in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS). Fractions containing the protein of interest were pooled and stored at -80 °C.

All recombinant polypeptides were analyzed by N-terminal sequencing (Applied Biosystems 475A or 494) resulting in the correct sequence for rF18-E1073K (APLADIDEXEV), rF45-wt (APLADYXQLVXYL), and rF45-N548I (APLADYXQLVRYLXQ). rF18-wt was sequenced previously (6). Production, purification, and characterization of constructs rF11 and rF6trunc were performed as described in detail previously (6). To test for correct folding, purified wild-type and mutated polypeptides were analyzed by standard Western blotting techniques as described in detail (28). Equal aliquots (4.2 μg) of each protein were subjected to SDS electrophoresis in the presence or absence of 5 mM dithiothreitol, transferred to nitrocellulose, and incubated either with ~10 μg/ml monoclonal antibody 201 (rF45-wt, rF45-N548I), or a 1:200 dilution of polyclonal antiserum B9543 (rF18-wt, rF18-E1073K). These mono- and polyclonal antibodies are specific for authentic fibrillin-1 and do not react with reduced material.

Secretion of Recombinant Proteins from Cell Clones—Confluent layers of recombinant cells were incubated in 80-cm² flasks with 10 ml of serum-free culture medium for 48 h. The medium was harvested and after washing the cell layer twice with phosphate-buffered saline, the cells were solubilized in 1 ml of SDS sample buffer. Equal relative amounts of the serum-free medium (0.25 ml after precipitation with

Fibrillin-1

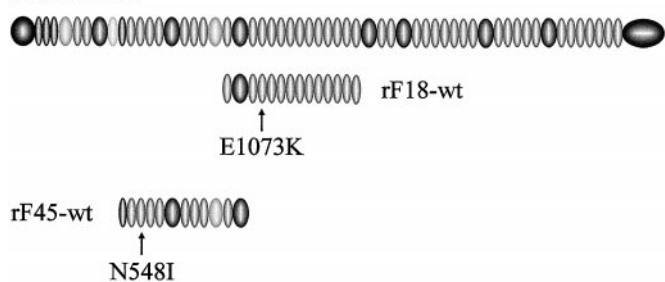


Fig. 1. Schematic drawing of recombinantly expressed polypeptides. The location of mutations N548I and E1073K within the wild-type polypeptides are indicated.

10% trichloroacetic acid) and the cell layer (25 μl) were analyzed by standard Western blotting techniques as described in detail (28). Blotted proteins were incubated with ~10 μg/ml monoclonal antibody 201 (rF45-wt, rF45-N548I), or 1:1000 diluted monoclonal antibody anti-His (C-term) (Invitrogen), which recognizes the histidine tag at the C-terminal end of rF18-wt and rF18-E1073K.

Degradation Experiments and Sequence Analysis—Recombinant polypeptides were dialyzed against TBS, adjusted to a concentration of 1 mg/ml (rF18-wt, rF18-E1073K), 1.4 mg/ml (rF45-wt, rF45-N548I), or 0.93 mg/ml (rF11, rF6trunc), and supplemented with 5 mM CaCl₂ or 5 mM EDTA (10 mM CaCl₂ or 10 mM EDTA for rF11 and rF6trunc). After a 10-min equilibration period, an aliquot was removed for a zero time control. Enzymes were added at a concentration of 1:20–1:100 (w/w) (plasmin, EC 3.4.21.7, Roche Molecular Biochemicals), or 1:100–1:500 (w/w) (trypsin, EC 3.4.21.4, treated with tosylphenylalanyl chloromethyl ketone; α-chymotrypsin, EC 3.4.21.1, treated with N^α-p-tosyl-L-lysyl chloromethyl ketone; Sigma; endoproteinase Glu-C, EC 3.4.21.19, Roche Molecular Biochemicals) for incubation periods of 0–22 h (plasmin) or 0–60 min (trypsin, chymotrypsin, and endoproteinase Glu-C). The reaction was stopped by adding 2-fold concentrated reducing SDS sample buffer to equal aliquots of the samples and heating at 95 °C for 3 min. Degradation products were separated by SDS-gel electrophoresis with homogenous gels containing 7.5–12% (w/v) acrylamide and visualized by Coomassie staining.

For N-terminal sequence analysis, the degradation products were transferred to a PVDF membrane (Immobilon-P, Millipore or ProBlott, Applied Biosystems), stained by Coomassie Blue, excised, and analyzed on a protein sequencer (Applied Biosystems Model 475 or 494).

Circular Dichroism Measurements—The purified recombinant polypeptides were dialyzed against TBS, supplemented with 5 mM CaCl₂ or 0.2 mM EDTA, and diluted to concentrations of 0.375 mg/ml. Spectra from 190 to 260 nm were recorded in a 1-mm quartz cuvette at 20 °C on a Jasco J-715 instrument.

Electron Microscopy—Purified polypeptides were adjusted to a concentration of 0.15 mg/ml, supplemented with 5 mM CaCl₂, and dialyzed against H₂O. The samples were diluted to a final concentration of 70% (v/v) glycerol, sprayed onto freshly cleaved mica, and dried under vacuum (Edwards Auto 306). Rotary shadowing was performed basically as described with minor modifications (29). Replicas were examined at 80 kV in a transmission electron microscope (Zeiss TEM 109).

RESULTS

We investigated the structural and functional consequences of two mutations in fibrillin-1, leading to the classical form (N548I) and the “neonatal” form (E1073K) of Marfan syndrome. Both mutations alter critical residues within cbEGF modules that have been shown to be directly involved in calcium binding (30). The N548I mutation was introduced into a new fibrillin-1 wild-type construct (rF45-wt, Asp⁴⁵¹-Lys¹⁰²⁷, Fig. 1) spanning the fourth generic EGF-like module to the third 8-Cys module. The E1073K mutation was introduced into the previously described rF18 construct (rF18-wt, Asp⁹¹⁰-Val¹⁵²⁷, Fig. 1) spanning cbEGF10 to cbEGF22 (6). Wild-type and mutated polypeptides were expressed and efficiently secreted from human embryonic kidney cells in amounts of ~10–20 μg/ml/day into the culture medium. Each recombinant construct was exclusively detected after a 48-h incubation pe-

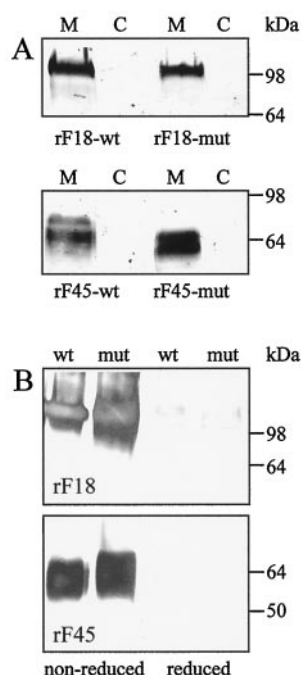


FIG. 2. **Immunochemical tests for secretion and folding of recombinant polypeptides.** A, analysis of wild-type (rF18-wt, rF45-wt) and mutated (rF18-mut, rF45-mut) polypeptides in the serum-free culture medium (M) and the cell layer (C) by Western blotting with specific antibodies (see “Experimental Procedures” for details). Note that no recombinant protein has been observed in the cell layer. B, analysis of purified recombinant wt and mutated (mut) polypeptides by Western blotting under nonreducing and reducing conditions. Polyclonal antiserum B9543 was used for the rF18 polypeptides and monoclonal antibody 201 was used for the rF45 polypeptides. Note that these antibodies only react with the nonreduced but not with the reduced forms of the recombinant polypeptides.

riod in the cell culture medium and virtually no recombinant protein was retained within the cells (Fig. 2A).

Structural Properties of Wild-type and Mutated Polypeptides—In Western blotting experiments, the wild-type and mutated polypeptides bound strongly either to monoclonal antibody 201 (rF45-wt and rF45-N548I) or to polyclonal antibodies B9543 (rF18 and rF18-E1073K), whereas reduced material did not bind (rF45-wt, rF45-N548I) or bound only very weakly (rF18-wt, rF18-E1073K) to the corresponding antibodies (Fig. 2B). These immunochemical tests clearly indicated that the recombinant polypeptides are folded correctly, since epitope recognition of these antibodies depends on intact disulfide bonds. For subsequent structural and functional analyses, the wild-type and mutated polypeptides were purified from conditioned medium to homogeneity.

Electron microscopy after rotary shadowing demonstrated, as expected from the shape of full-length fibrillin-1, long extended molecules for the wild-type polypeptides (Fig. 3A). This typical extended shape is another good indicator for correct folding, since this shape is dependent on calcium binding and correct disulfide bonds (31, 32). No differences of the overall shape between the wild-type and the mutated forms were apparent (Fig. 3A). Measurements of the lengths of well resolved individual particles revealed a small but significant longer shape for rF18-wt ($35.4 \text{ nm} \pm 1.6 \text{ S.D.}$; $n = 60$) as compared with rF18-E1073K ($33.7 \text{ nm} \pm 2.1 \text{ S.D.}$; $n = 60$), and virtually identical lengths for rF45-wt ($27.9 \text{ nm} \pm 1.8 \text{ S.D.}$; $n = 50$) and rF45-N548I ($27.5 \text{ nm} \pm 2.3 \text{ S.D.}$; $n = 50$) (Fig. 3B).

Analyses of the constructs by far UV circular dichroism revealed slightly different spectra for the wild-type and the mutated polypeptides (Fig. 4). In the presence of calcium, minima

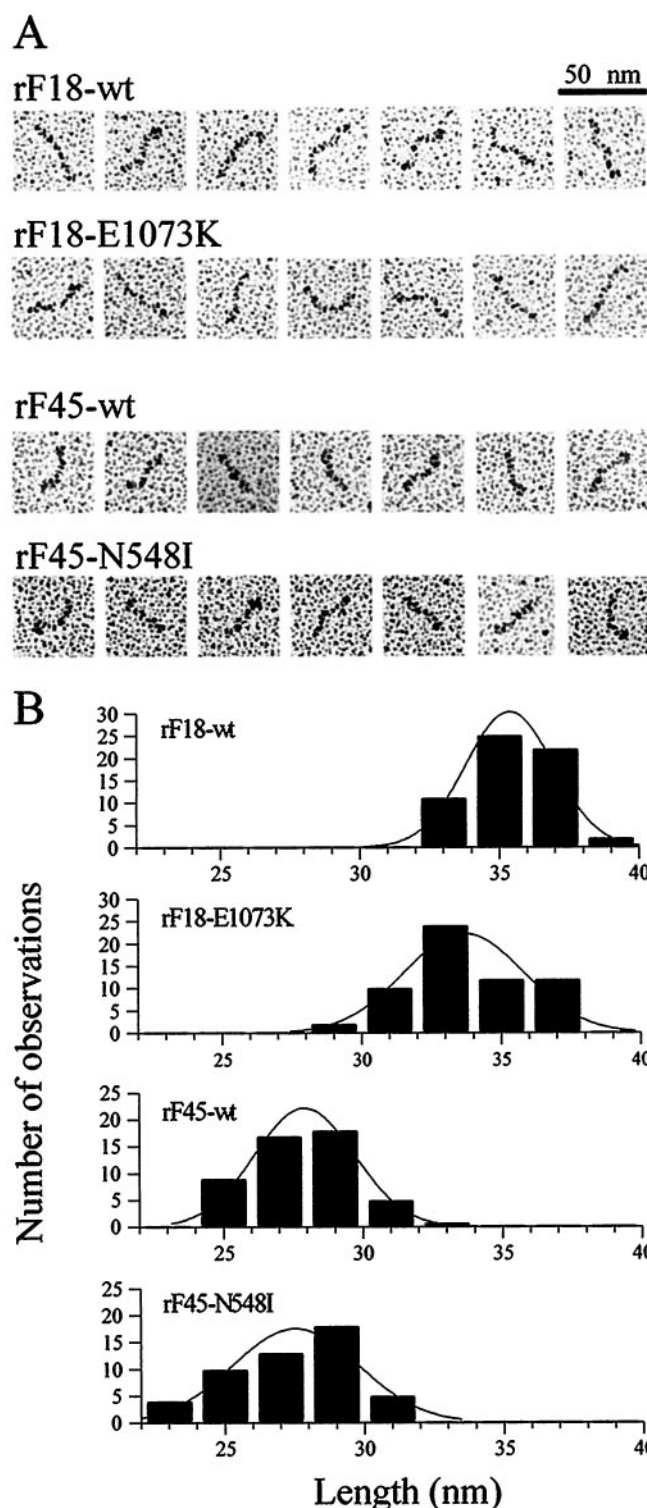


FIG. 3. **Shape and length of wild-type and mutated polypeptides.** A, electron microscopic images after rotary shadowing. Note that mutations N548I and E1073K do not cause a gross structural change of the molecules. B, histogram of measured lengths of the recombinant polypeptides. Measurements are plotted as numbers of measurements in 2-nm windows (bars). Gaussian curves for measurements of each polypeptide are shown (curves).

were observed for rF18-wt ($\theta = -7169 \text{ degree cm}^2 \text{ dmol}^{-1}$) at 212 nm and rF18-E1073K ($\theta = -6385 \text{ degree cm}^2 \text{ dmol}^{-1}$) at 210 nm (Fig. 4A), and for rF45-wt ($\theta = -6908 \text{ degree cm}^2 \text{ dmol}^{-1}$) at 209 nm and rF45-N548I ($\theta = -5649 \text{ degree cm}^2 \text{ dmol}^{-1}$) at 208 nm (Fig. 4B). In the presence of EDTA, the

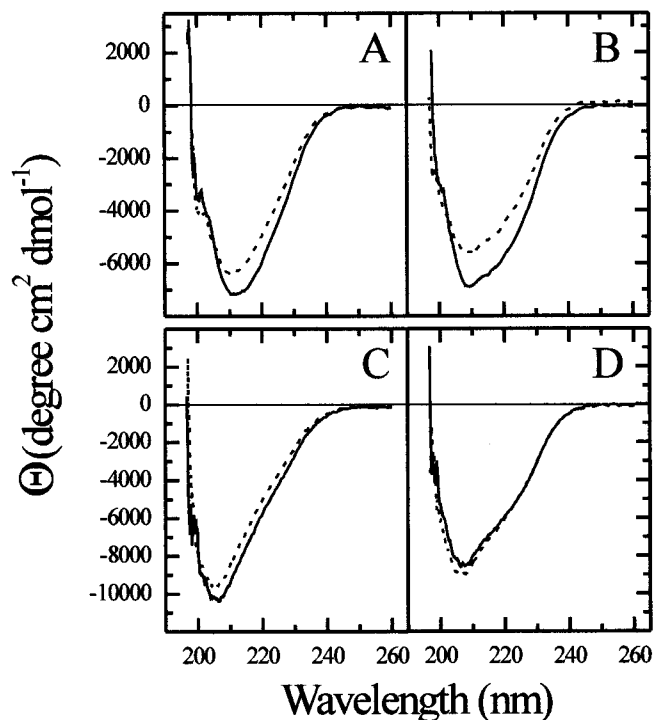


FIG. 4. Far UV circular dichroism spectra of wild-type and mutated polypeptides. The experiment was performed in the presence of 5 mM CaCl_2 (A and B) or 0.2 mM EDTA (C and D) at 20 °C with 0.375 mg/ml (A and C) rF18-wt (solid line), rF18-E1073K (dotted line), or (B and D) rF45-wt (solid line) and rF45-N548I (dotted line). Measurements at wavelengths below ~198 nm are not shown due to strong absorption of the buffer. Ellipticity θ is plotted as a function of wavelength.

minima decreased to $\theta = -10409$ degree $\text{cm}^2 \text{dmol}^{-1}$ (rF18) and $\theta = -9659$ degree $\text{cm}^2 \text{dmol}^{-1}$ (rF18-E1073K) at 206 nm (Fig. 4C), and to $\theta = -8542$ degree $\text{cm}^2 \text{dmol}^{-1}$ (rF45), and $\theta = -8978$ degree $\text{cm}^2 \text{dmol}^{-1}$ (rF45-N548I) at 208 nm (Fig. 4D). These data indicate that small changes in the secondary structures occur upon introduction of N548I or E1073K into the corresponding constructs.

Additional analyses by SDS-polyacrylamide gel electrophoresis demonstrated for both wild-type and mutated polypeptides identical gel retention of ~120 kDa for the rF18 polypeptides (Fig. 5, left panels) and ~80 kDa for the rF45 polypeptides (Fig. 6, left panel).

Proteolytic Degradation of Wild-type and Mutated Polypeptides—The recombinant polypeptides were extensively analyzed for their susceptibility to a variety of proteases. rF18 and rF18-E1073K were incubated with trypsin, chymotrypsin, endoproteinase Glu-C, and plasmin (Fig. 5). In the presence of calcium, rF18-wt was resistant to proteolytic cleavage. rF18-E1073K showed degradation products of about 70–80 and 20 kDa even after very short incubation times (e.g. 2 min for incubation with chymotrypsin), indicating that rF18-E1073K is much more susceptible to proteolytic degradation as compared with rF18-wt (Fig. 5A). When rF18-wt and rF18-E1073K were incubated with proteases in the presence of EDTA, no differences in degradation patterns have been observed (Fig. 5B). Both constructs were degraded significantly faster in the absence of calcium as observed previously with other fibrillin-1 subdomains (11). Proteolytic degradation products of rF18-E1073K which were absent in rF18-wt (marked by arrows and letters in Fig. 5) were analyzed by N-terminal sequencing (Table I). All three tryptic sites were within the mutated cbEGF12 either close to the mutation (at position 1076) or somewhat

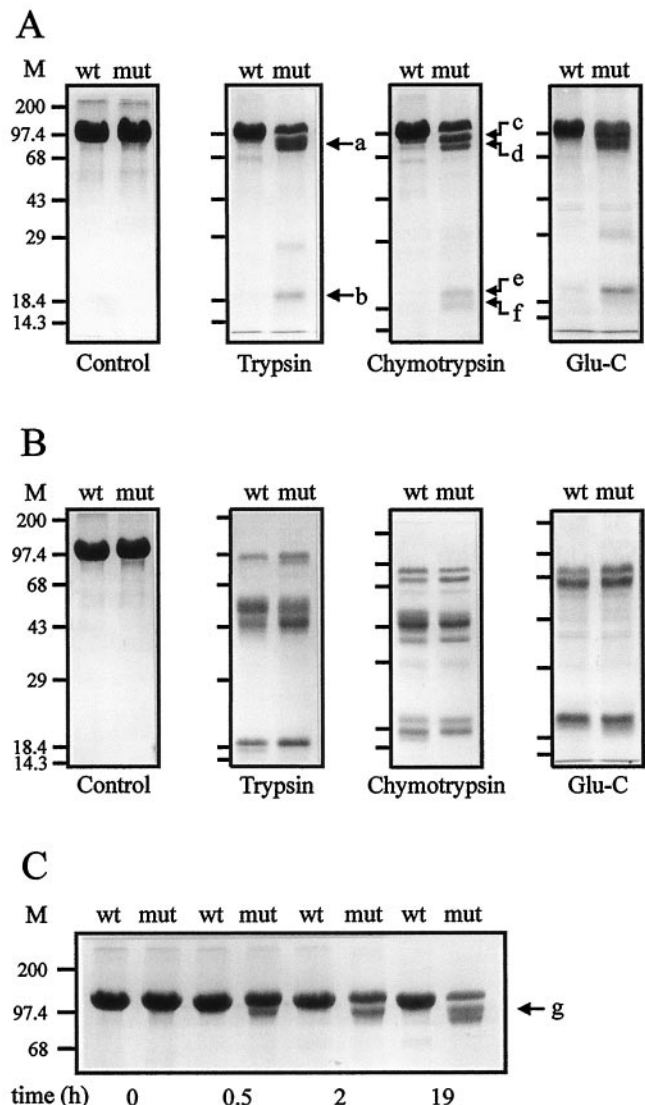


FIG. 5. Degradation of rF18-wt (wt) and rF18-E1073K (mut) by various proteases. The recombinant polypeptides were treated in the presence of 5 mM CaCl_2 (A) or 5 mM EDTA (B) with trypsin (5 min), chymotrypsin (5 min), or endoproteinase Glu-C (Glu-C, 60 min) at an enzyme:substrate ratio of 1:100 (w/w). In C, the polypeptides were treated in the presence of 5 mM CaCl_2 with plasmin (enzyme:substrate = 1:50 (w/w)) for incubation times as indicated. Small ~15–20 kDa degradation products close to the running front of the gel in C are not shown. The degradation products were analyzed by Coomassie Blue staining after SDS-gel electrophoresis. Proteolytic degradation products indicated by an arrow and a letter were further analyzed by N-terminal sequencing (see Table I and Fig. 7). The molecular masses of reduced marker proteins are indicated in kDa (M).

more C-terminal (at positions 1084 and 1110). Although the E1073K substitution introduced a new residue for potential tryptic attack, no cleavage site were observed at position 1073. Sensitive sites produced with chymotrypsin were observed in the last loop of the preceding cbEGF11 at position 1060 or within the last loop of the mutated cbEGF12 at positions 1107 and 1109. The ~20–25-kDa fragments were identified as the N termini of the respective polypeptides. Degradation with plasmin revealed a sensitive site N-terminal of the preceding cbEGF11 at position 1028, indicating that a structural change must be conveyed beyond the length of one cbEGF module.

Similar results were obtained with rF45-N548I. Incubation of this polypeptide with trypsin, chymotrypsin, and plasmin in the presence of calcium demonstrated significantly enhanced susceptibility to proteolytic degradation as compared with the

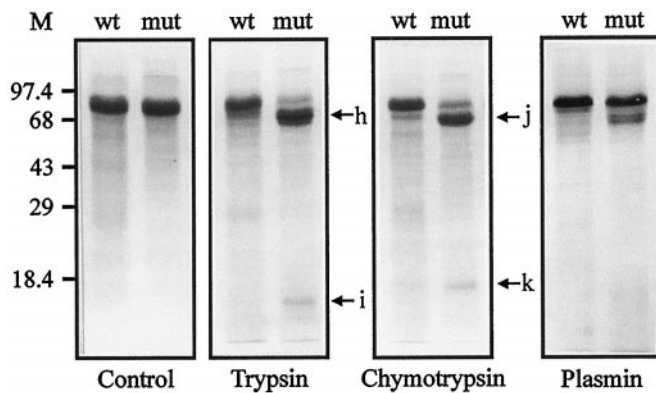


FIG. 6. **Degradation of rF45-wt (wt) and rF45-N548I (mut) by various proteases.** The recombinant polypeptides were treated in the presence of 5 mM CaCl_2 with trypsin or chymotrypsin (enzyme:substrate = 1:100 (w/w), 2 min), or plasmin (enzyme:substrate = 1:20 (w/w), 22 h), and analyzed by SDS-gel electrophoresis and Coomassie Blue staining. Proteolytic degradation products which were further analyzed by N-terminal sequencing are marked with an arrow and a letter (see Table I and Fig. 7). Positions of reduced marker proteins are indicated in kDa (M).

wild-type form (Fig. 6). In all cases, the full-length rF45-N548I (~80 kDa) is rapidly degraded into 60–65- and 15–20-kDa fragments. In the absence of calcium, degradation progressed much faster and no differences in protease susceptibility between wild-type and mutated forms were observed (data not shown). Sequence analyses of the larger proteolytic fragments generated with trypsin and chymotrypsin resulted in identification of sites at positions 526 and 566 (trypsin) and 524 and 562 (chymotrypsin) (Table I). These cleavage sites are located relatively close to the altered amino acid residue in the last loops of cbEGF3 and the mutated cbEGF4. The intensities of the 15–20-kDa degradation products are weaker than expected from the intensities of the 60–65-kDa fragments (Fig. 6, band *i* and *k*). This may indicate additional sensitive sites within these N-terminal fragments created by longer range structural effects of the N548I mutation.

The sensitive cleavage sites found in the mutated polypeptides but not in the wild-type forms are schematically mapped onto cbEGF11-cbEGF12 (rF18-E1073K) in Fig. 7A, and onto cbEGF3-cbEGF4 (rF45-N548I) in Fig. 7B.

To test whether plasmin is able to degrade other regions of non-mutated fibrillin-1, we tested previously characterized recombinantly expressed fibrillin-1 subdomains rF11 and rF6trunc (6) in degradation experiments with plasmin (Fig. 8). As observed with other proteases before (11), these polypeptides are significantly stabilized by calcium against plasmin degradation and become susceptible to proteolysis when calcium is removed by EDTA.

DISCUSSION

Previously, we demonstrated that calcium significantly protects fibrillin-1 against proteolytic degradation by a variety of proteases (11). Based on these results, we hypothesized that mutations in fibrillin-1 that alter residues involved in calcium binding would render the fibrillin-1 molecules more susceptible to proteolysis in the mutated region. Enhanced degradation of monomeric or polymeric molecules would then lead over time to fewer or shorter microfibrils or mechanically weakened microfibrils (11). These effects on microfibrils would then result in disease.

Here we have described the first experiments to test this hypothesis. We have generated two recombinant fibrillin-1 subdomains harboring Marfan mutations in cbEGF modules that are predicted to be directly involved in calcium binding (30).

TABLE I
Degradation products of mutant polypeptides
Degraded protein fragments indicated in Figs. 5 and 6 (code) were analyzed by N-terminal sequencing.

Protease	Code	N-terminal sequence	Position
rF18-E1073K			
Trypsin	a	ISPDLXGR(G)(Q)	1076
		GQXVN(T)PGX(F)	1084
		NXMDIXE	1110
Chymotrypsin	b	APLADIDEXE	N terminus
	c	ALDSEER	1060
	d	MMKNX(M)DI(D)E	1107
	e	KNXMDIDEXQ	1109
		APLADIDE	N terminus
Plasmin	f	APLAD	N terminus
	g	DINEXKMIP(S)LX(T)	1028
rF45-N548I			
Trypsin	h	DGKNXEDMD(E)	566
		TEXRDIDEXL	526
	i	AP(L)ADYXQLV	N terminus
Chymotrypsin	j	TRTEXRDIDE	524
	k	(H)VTXDGKNXE	562
		APLADYXQLV	N terminus

One mutation (N548I) leads to the classical form of the Marfan syndrome (27) and the second mutation (E1073K) leads to the more severe neonatal form of this disorder (24, 25). Wild-type and mutated polypeptides were expressed and secreted in similar amounts from recombinant mammalian cells into the culture medium, and virtually no recombinant protein was retained within the cells. These results demonstrate that neither mutation had any major inhibiting effect on synthesis and secretion of the recombinant polypeptides. This is consistent with data available for secretion of fibrillin-1 harboring the N548I mutation from patient cells (33).

Although native folding of the large recombinant wild-type polypeptides cannot be proven directly, two lines of evidence provide strong support for correct folding: (i) the epitopes for mono- and polyclonal antibodies that require intact disulfide bonds are present, and (ii) the polypeptides adopt an extended thread-like conformation as expected from the shape of authentic fibrillin-1 (34). In addition, the mammalian expression system used has previously produced a number of similar recombinant fibrillin-1 polypeptides, which have been demonstrated by a variety of methods to be folded correctly (6, 10, 11, 28, 32). Both mutations did not alter antibody recognition or the extended conformations, indicating that the overall fold of the polypeptides is not affected upon substitution of N548I or E1073K. However, structural analyses by electron microscopy after rotary shadowing and comparison of far UV circular dichroism spectra revealed small structural changes of the mutated polypeptides rF18-E1073K and rF45-N548I compared with the wild-type forms rF18-wt and rF45-wt. Extensive protease degradation assays with a variety of proteases showed that both of the mutated polypeptides are significantly more susceptible to proteolytic damage compared with the non-mutated forms. Since the cleavage sites are exclusively located relatively close to the mutated residues, we conclude that the structural changes observed are localized in these regions.

It is likely that the mutations alter calcium binding affinities of the mutated cbEGF modules, since they substitute crucial residues for these interactions. It is not feasible, however, to experimentally determine the potential reduction or complete loss of calcium binding to the mutated cbEGFs within these polypeptides, due to the total numbers of calcium-binding sites

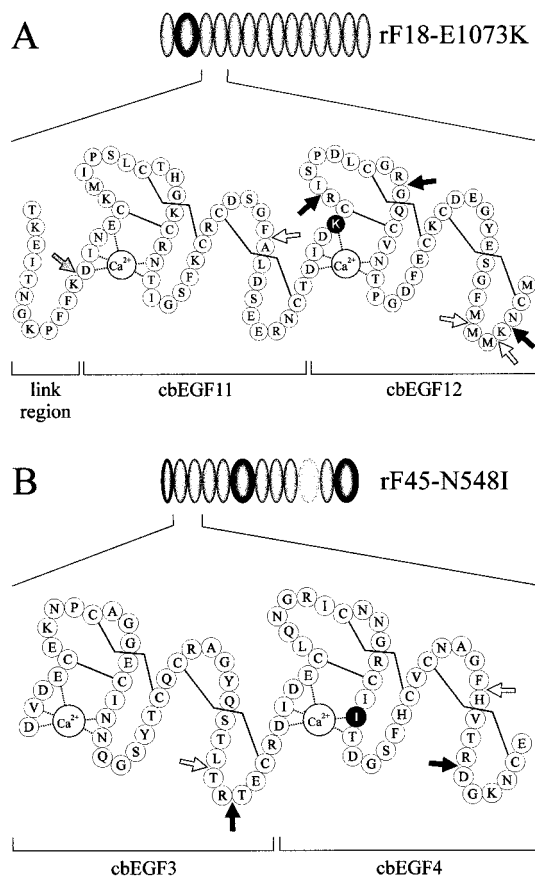


FIG. 7. Schematic drawing of rF18-E1073K (A) and rF45-N548I (B). The upper parts of each figure show the modular arrangement of the entire subdomains. The positions of these subdomains relative to the entire fibrillin-1 molecule can be seen in Fig. 1. Two cbEGF modules including the mutations are enlarged for each subdomain. Amino acid residues are shown as circles and mutations are indicated in black. Sensitive sites (see Table I, Figs. 5 and 6) produced with trypsin (black arrows), with chymotrypsin (white arrows), and plasmin (gray arrow) are mapped within the cbEGF modules.

present (8 and 13), and to various calcium affinities of individual cbEGF modules (7).² Despite this limitation, the following line of evidence suggests that the enhanced susceptibility for proteases is associated with a local loss of calcium binding. In the presence of calcium, only the regions close to the mutations are significantly degraded whereas most non-mutated cbEGF modules are protected. Similar enhanced proteolytic degradation patterns have been observed previously with other wild-type fibrillin-1 cbEGF modules when calcium was removed by EDTA (11). Finally, wild-type and mutated polypeptides were equally susceptible to proteolytic degradation in the absence of calcium. Reduced calcium binding affinities have been directly observed as a consequence of another mutation, N2144S, using small synthetic or bacterially expressed peptides (4, 23). This mutation substitutes an asparagine residue in cbEGF32 at an equivalent position to the N548I mutation in cbEGF4. In that study (4), a fibrillin-1 peptide of cbEGF32 with the N2144S substitution showed 5-fold decreased calcium binding affinity as compared with the wild-type peptide. The N2144S mutation even further decreased the calcium affinity of cbEGF32 (9-fold) when this module was expressed in combination with a preceding 8-Cys module (23). The calcium binding affinity of the subsequent cbEGF33, however, was not altered, which demonstrated a locally restricted effect of this mutation (23).

² D. P. Reinhardt, unpublished observations.

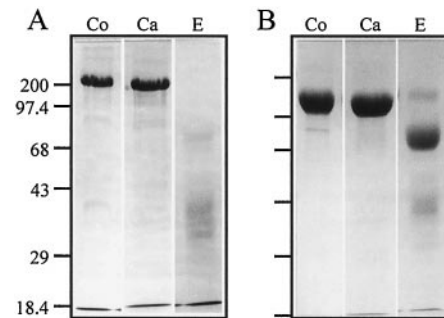


FIG. 8. Degradation of (non-mutated) fibrillin-1 polypeptides rF11 (A) and rF6trunc (B) with plasmin. The subdomains were treated in the presence of 10 mM CaCl_2 (Ca) or 10 mM EDTA (E) with plasmin (enzyme:substrate = 1:100) for 17.5 h and then analyzed by gel electrophoresis and Coomassie staining. The control (Co) was not incubated with plasmin. Control incubations for up to 21 h in the absence of plasmin did not result in fragmentation of the recombinant polypeptides. Molecular masses of marker proteins are indicated in kDa.

In the present study, some sensitive sites were observed close to the mutated residue E1073K in the N-terminal calcium-binding region of cbEGF12 in cysteine loops 1–3. These sensitive sites can be explained either by reduced steric hindrance for the proteases caused by a missing calcium ion or by a structural change in this region introduced by the substitution of a basic residue (lysine) for an acidic residue (glutamic acid). Other sensitive sites caused either by N548I or E1073K are located at more distance from the mutations in cysteine loops 5–6 of the mutated (cbEGF4 and -12) or the preceding (cbEGF3 and -11) modules. These data demonstrate that the structural effects of the mutations are not only confined to the immediate vicinity of the mutated residues. Furthermore, when plasmin was used to degrade rF18-E1073K, the first cleavage product found started at position Asp¹⁰²⁸, which is located N-terminal to cbEGF11, preceding the mutated cbEGF12. This clearly demonstrates a longer range (~3 nm) structural effect of the E1073K mutation that is not confined to the mutated cbEGF module or to immediately adjacent regions. Possibly, longer range structural effects also occur in rF45-N548I, since the intensity of the N-terminal proteolytic fragments do not correspond to the intensities of the large degradation products, indicating that additional sensitive sites are located N-terminal to the cleavage site (see Fig. 6). Longer range stabilizing effects of calcium binding has been reported in cbEGF modules of human Notch-1 protein (8). Tandemly repeated cbEGF modules in fibrillin-1 are separated by one amino acid residue (class I), whereas repeats in Notch-1 are separated by two residues (class II) (31). Thus, transmission of longer range effects does not appear to be dependent on the length of the linker region between cbEGF repeats. The structural basis for how these changes are communicated over an entire intervening domain remains to be established.

The current dominant-negative model of Marfan syndrome pathogenesis requires that mutated fibrillin-1 is expressed above a certain threshold of about 6–15% of that expressed from the wild-type allele (35, 36). The mutated molecules must have the ability to disrupt normal functions of fibrillin-1 on some level. On the other hand it was shown that reduced expression levels of normal fibrillin-1 in mice lead to a phenotype similar to Marfan syndrome with aortic aneurysm and dissection (37). The consequences in both situations would be a reduced amount of correctly functional microfibrils in the extracellular matrix. How multiple different mutations in fibrillin-1 can each cause the dominant negative effects in Marfan syndrome is largely obscure on the molecular level. Quantita-

tive pulse-chase analyses of fibrillin-1 from a variety of patient cells revealed either defects in the synthesis, secretion, and matrix deposition of fibrillin-1, or no defects at all (33, 38). Although in most of these cases the corresponding fibrillin-1 mutations were not known, one can roughly assume that point mutations in cbEGF modules represent close to 50% of these mutations (see Introduction). Enhanced proteolytic degradation of mutated fibrillin-1 could help explain how these types of mutations exert their deleterious effects on several levels. Degradation of mutated fibrillin-1 could occur as individual molecules transit through the secretory pathway, or during the first steps of assembly in the extracellular matrix, or after the molecules have been incorporated into microfibrils. Proteolytic degradation could affect important ligand-binding sites, either through direct disruption of a binding site or through longer range effects, and thus disturb assembly by disruption of self-assembly sites or sites for other protein ligands important in the assembly process. If mutated fibrillin-1 becomes proteolytically degraded when it is already incorporated into the microfibril, then it is conceivable that these cleavage sites represent mechanically weak spots within the microfibrils.

Several other mutations of equivalent residues to the E1073K substitution (E1200G and E2447K) or to the N548I substitution (N1131Y, N1173K, N1382S, and N2144S) have been reported in other cbEGF modules of fibrillin-1 (13). The clinical phenotypes of these mutations are very different. For example, the E2447K mutation results in isolated ectopia lentis (39), E1200G in classical Marfan syndrome, while E1073K leads to neonatal Marfan syndrome (24, 25). On the other hand, N1131Y results in neonatal Marfan syndrome (40), while N548I was found in an individual with classical Marfan syndrome (27). Proteolytic degradation of fibrillin-1 at these different mutated sites resulting in different molecular consequences could help explain the development of these various clinical phenotypes.

Whether or not a mutated fibrillin-1 molecule becomes degraded depends on the availability of recognition sites for proteases that are exposed after structural changes are introduced by a given mutation. Plasmin, identified here as a fibrillin-1 degrading enzyme, could play a role in potential degradation of mutated fibrillin-1 in a pathological situation since (i) it has wide substrate specificity and could thus attack a wide variety of mutated sites and (ii) it is available in extracellular matrices at sites where fibrillin-1 and microfibrils are expressed (41). In the mouse *fbn1* gene-targeting experiment, pathogenesis of aortic disease involved smooth muscle cell proliferation and macrophage infiltration (37). Thus, metalloproteases secreted by smooth muscle cells or inflammatory cells may also represent decisive elements for potentially enhanced fibrillin-1 degradation as disease progresses. If indeed proteolytic degradation of mutated fibrillin-1 plays an important role in the pathogenesis of Marfan syndrome and other disorders caused by mutations in cbEGF modules, then the identification of the relevant proteases and protease inhibitors may lead to the development of new therapeutic strategies.

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Mutations in Calcium-binding Epidermal Growth Factor Modules Render Fibrillin-1 Susceptible to Proteolysis: A POTENTIAL DISEASE-CAUSING MECHANISM IN MARFAN SYNDROME

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